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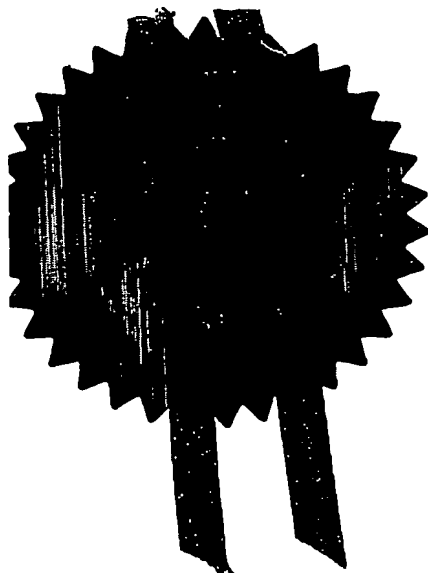
PCT

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1. Your reference

P31283-JDU/BOU

2. Patent application number

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0216371.5

13 JUL 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Rowett Research Institute
Greenburn Road
Bucksburn
Aberdeen
AB21 9SB

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

4151262001

4. Title of the invention

"Compounds"

5. Name of your agent (*if you have one*)

"Address for service" in the United Kingdom to which all correspondence should be sent (*including the postcode*)

Murgitroyd & Company
165-169 Scotland Street
GLASGOW
G5 8PL

Patents ADP number (*if you know it*)

1198013

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (*if you know it*) the or each application number

Country

Priority application number
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Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (*Answer 'Yes' if:*

Yes

- a) any applicant named in part 3 is not an inventor, or
 - b) there is an inventor who is not named as an applicant, or
 - c) any named applicant is a corporate body.
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Continuation sheets of this form

Description

28

Claim(s)

Abstract

Drawing(s)

4 + 4

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10. If you are also filing any of the following, state how many against each item.

Priority documents

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature *Murgitroyd & Co.*

Date
12 July 2002

Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom

0141 307 8400

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1 Compounds

2

3 The present invention relates to new analogues of
4 phytochemicals, to compositions comprising these
5 analogues and to the use of these analogues as
6 therapeutic agents.

7

8 Particularly but not exclusively the present
9 invention relates to new analogues of flavonoids
10 having improved lipid solubility and the ability to
11 orientate themselves within lipid membranes.

12

13 Oxidative damage to cells is implicated in the
14 development of many clinical conditions including
15 ischaemia-reperfusion injury, cancers, heart
16 disease, arthritis, neurological disorders and auto-
17 immune diseases. To date preventative therapy with
18 antioxidants has not been very successful, partly
19 because targeting and orientating the compounds at
20 the correct site within the cell for optimum effect
21 is difficult. Evidence is now emerging that
22 effective antioxidant intervention during the acute

1 phase of ischaemic events may increase survival rate
2 and minimise irreversible organ damage.

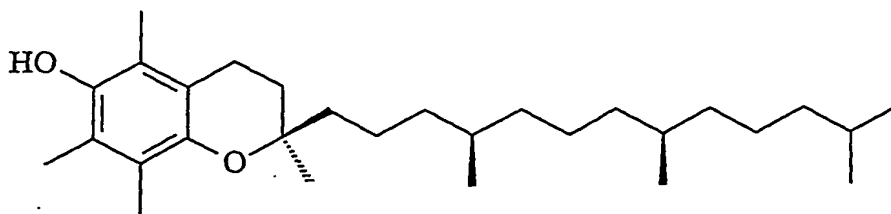
3
4 Combinational therapies for treatment of diseases
5 currently incorporate natural and synthetic
6 antioxidants with limited success. There is a need
7 to produce antioxidant agents that possess low
8 toxicity and high therapeutic benefit for use in
9 pharmaceutical preparations. Current natural
10 flavonoid antioxidants are relatively ineffective,
11 being inefficient at targeting molecules.

12
13 The low bioavailability and uptake by the human body
14 of dietary antioxidants is a limiting factor in
15 their therapeutic action. Dietary antioxidants have
16 poor performance in the treatment of diseases such
17 as Parkinson's and Alzheimer's and in ameliorating
18 ischaemia-reperfusion injury.

19
20 Vitamin E (d- α -tocopherol) is a widely used and
21 naturally occurring antioxidant. It is known to
22 protect cell membranes from free radical mediated
23 oxidative damage. The chemical structure of vitamin

24 E (d-(2R,4'R,8'R)- α -Tocopherol), is shown below;

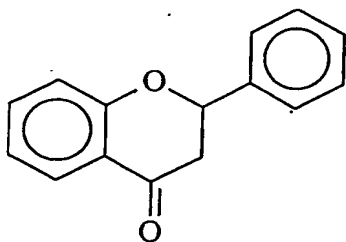
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1 The recognised essential dietary antioxidants are
2 vitamin E and vitamin C. There are also a range of
3 metals; including selenium, iron, copper, zinc and
4 manganese, required from the diet to allow
5 functioning the enzymes with antioxidant activities.
6 Carotenoids from the diet may also have antioxidant
7 properties *in-vivo* in the scavenging of singlet
8 oxygen and in tissues of low partial oxygen
9 pressure.

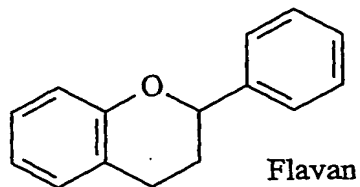
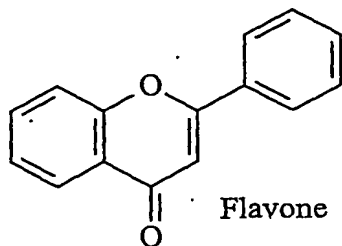
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11 Alternative natural antioxidants include flavonoids
12 which have the following general structure:

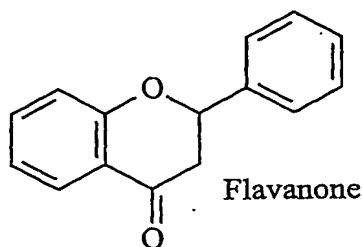
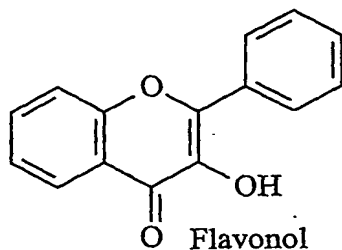


13 Flavonoids are polyhydroxyphenolic products of the
14 phenylpropanoid biosynthetic pathway in plants, and
15 there are more than 4000 naturally-occurring
16 flavonoids. They are present in a wide range of
17 fruits, vegetables, nuts, and beverages including
18 wine and tea. Flavonoids fall into two distinct
19 groups depending on whether the central heterocyclic
20 ring is saturated or unsaturated. If the central
21 heterocyclic ring is unsaturated (as in
22 anthocyanidin, flavones, flavonols), the molecule is
23 achiral. If the central heterocyclic ring is
24 saturated, as shown above, (as in flavanones and
25 flavans), one or more chiral centres are present,
26 and thus such flavonoids exhibit optical activity.
27 A number of flavonoid structures are shown below;

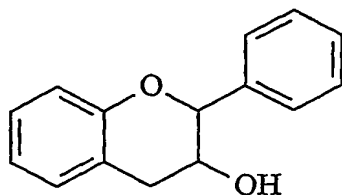
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2



3



4

5 Selected flavonoids, such as myricetin, exhibit
 6 potent antioxidant properties and are more effective
 7 as antioxidants than vitamin E both in terms of the
 8 number of radicals which one molecule can reduce and
 9 in terms of the rate of the radical annihilation
 10 reaction. However, flavonoids are poor membrane
 11 protectants due to their limited lipid solubility.
 12 Consequently flavonoids have had limited application
 13 as antioxidants *in vivo*.

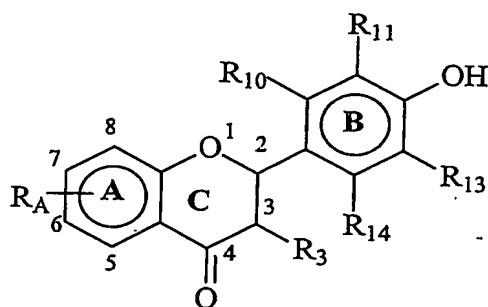
14

15 Our kinetic and stoichiometric studies comparing the
 16 reducing capabilities of flavonoids to a d- α -
 17 tocopherol indicate that the antioxidant activity is

markedly influenced by the number and position of the hydroxyl groups on the B and C rings as well as the extent of conjugation between the B and C rings. Moreover, within a biological system where a number of polyphenols may be present at similar concentrations, antioxidant efficacy may be predominantly governed by reaction kinetics rather than stoichiometry.

The present invention provides novel compounds having both potent antioxidant activity together with high lipid solubility, thus facilitating their sequestration into the cell membrane.

According to one aspect of the present invention there is provided a compound comprising a group R_A attached to the A ring of a flavonoid group of the following formula I:



Formula I

wherein;

R_A is a C_5 to C_{30} aliphatic alkyl chain; R_{10} , R_{11} , R_{13} , R_{14} , and R_3 each independently represent H, OH, or a C_1 to C_4 aliphatic alkyl; and

1 optionally there is a double bond between C₂ and
2 C₃ of the C ring.

3
4 Preferably at least one of R₁₀, R₁₁ and R₁₃ represents
5 OH. More preferably R₁₁ represents OH.

6
7 Suitably both R₁₁ and R₁₃ represent OH.

8
9 Preferably at least three of R₁₀, R₁₁, R₁₃, R₁₄ and R₃
10 represent OH.

11
12 Advantageously the flavonoid group is an extended
13 conjugated π -electron system.

14
15 Preferably there is a double bond between C₂ and C₃
16 of the C ring.

17
18 Preferably the B and C rings of the flavonoid have
19 the structure of the B and C rings of myricetin,
20 morin, quercetin, kaempferol, luteolin, or apigenin.
21 More preferably the B and C rings of the flavonoid
22 group have the structure of the B and C rings of
23 myricetin.

24
25 Alternatively the B and C rings of the flavonoid
26 group may have the structure of the B and C rings of
27 taxifolin or catechin.

28
29 R_A comprises an alkyl aliphatic backbone of from 5
30 to 30 carbon atoms. The backbone may be substituted
31 with small alkyl groups, such as CH₃ or C₂H₅.
32 Preferably the backbone of R_A has from five to

1 twenty carbon atoms, more preferably from eight to
 2 fifteen carbon atoms. The backbone may be saturated
 3 or unsaturated. Preferably the backbone is
 4 saturated.

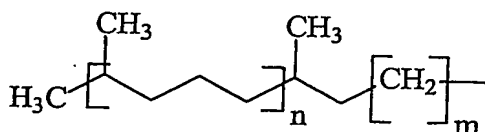
5

6 Suitably R_A is attached to position 5, 6, 7 or 8 of
 7 the A ring of the flavonoid group. Preferably R_A is
 8 attached to position 7 of the A ring of the
 9 flavonoid group.

10

11 In a preferred embodiment R_A has the following
 12 structure:

13



14

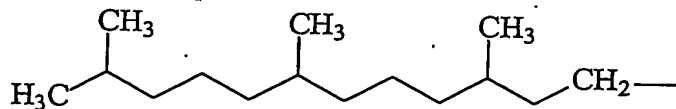
15 wherein

16 n is an integer from 1 to 7, preferably from 2
 17 to 3;

18 m is an integer from 1 to 7, preferably from 1
 19 to 2;

20

21 More preferably R_A has the following structure:



22

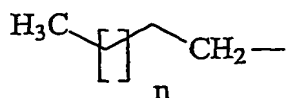
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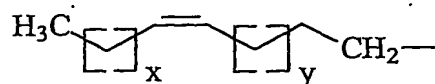
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1 Alternatively R_A has the following structure:



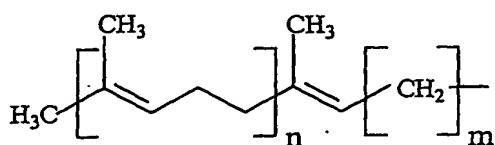
2
3 wherein n is an integer from 2 to 27, preferably n
4 is 2 to 17, more preferably 4 to 12.

5
6 In another embodiment R_A has the following
7 structure:



9
10 wherein
11 x is an integer from 1 to 25 preferably 1 to
12 15, more preferably 2 to 9;
13
14 y is an integer from 1 to 25, preferably 1 to
15 15, more preferably 2 to 9;
16
17 and wherein $x + y = 25$ or less.

18
19 ~~In another embodiment R_A has the following~~
20 ~~structure:~~



23

1 wherein

2 n is an integer from 1 to 7, preferably from 1
3 to 3, more preferably 2;
4 m is an integer from 1 to 7, preferably from 1
5 to 3, more preferably 2.
6

7 Whilst the Applicant does not wish to be bound by
8 theoretical considerations, it is believed that
9 addition of R_A to the A-ring increases membrane
10 partitioning and also adds the important spatial
11 distribution factor observed with vitamin E. It is
12 anticipated that crossing of the blood/brain barrier
13 will also be enhanced.
14

15 According to a further aspect of the present
16 invention there is provided a composition comprising
17 a compound as described above and at least one
18 pharmaceutically acceptable excipient or carrier.

19 The composition may be a sunscreen composition.

20 According to a further aspect of the present
21 invention there is provided a method of preventing
22 UV damage to the skin (for example sunburn or skin
23 cancers such as melanoma) comprising the step of
24 administering a therapeutically effective amount of
25 the sunscreen composition as described above to a
26 patient.
27

28 The composition will usually be applied topically.
29

30 The composition may alternatively be formulated as a
31 skincare composition and may, for example, include
32 emollients and moisturisers. The skincare

1 composition may be of particular utility in
2 preventing or reversing the effects of ageing, of
3 reducing apparent wrinkling, and/or treating or
4 preventing dry skin.

5
6 According to a further aspect of the present
7 invention there is provided a foodstuff stabiliser
8 composition comprising a compound as described
9 above.

10

11 It is believed that the ability to combat free
12 radicals will be of utility in preventing or
13 delaying the deterioration in food quality during
14 storage. It is envisaged that the composition will
15 be particularly effective where the foodstuff
16 stabiliser composition is in the form of an
17 emulsion, especially an emulsion having a low
18 fat/high water content. The foodstuff stabiliser
19 composition will be particularly suitable for low
20 fat spreads, salad dressings etc.

21 According to a further aspect of the present
22 invention there is provided a method of treating a
23 patient having a disease or disorder involving

24 ~~oxidative damage, said method comprising the step of~~
25 administering a therapeutically effective amount of
26 the composition described above to said patient.

27

28 The disease or disorder involving oxidative damage
29 may be selected from the group consisting of cancer
30 (for example colon, liver or bladder cancer), heart
31 disease, especially to prevent subsequent heart
32 attacks, neurological disorders, (particular mention

1 may be made of Alzheimer's or Parkinson's disease),
2 auto-immune disorders (particularly arthritis),
3 ischaemia-reperfusion injury, diabetic
4 complications, septic shock, hepatitis,
5 atherosclerosis and complications arising from HIV
6 or Hepatitis B.

7

8 Most suitably the disease to be treated is an
9 ischaemia-reperfusion injury.

10

11 According to a further aspect of the present
12 invention there is provided the use of a compound as
13 described above for the manufacture of a medicament
14 for the treatment of a disease or disorder involving
15 oxidative damage.

16

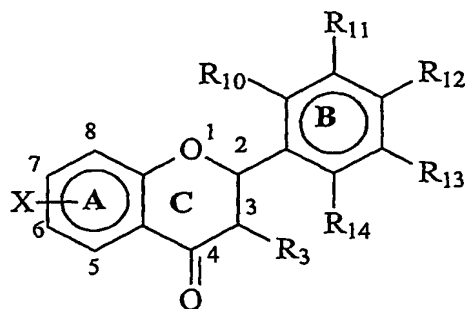
17 Suitably the disease or disorder may be cancer (for
18 example colon, liver or bladder cancer), heart
19 disease, especially to prevent subsequent heart
20 attacks, neurological disorders, (particular mention
21 may be made of Alzheimer's or Parkinson's disease),
22 auto-immune disorders (particularly arthritis),
23 ischaemia-reperfusion injury, diabetic
24 complications, septic shock, hepatitis,
25 atherosclerosis, and complications arising from an
26 immune response to HIV or Hepatitis B. Most
27 suitably the disease or disorder is ischaemia-
28 reperfusion injury.

29

30 The composition described above may be used
31 prophylactically or curatively.

32

1 According to a further aspect of the present
 2 invention there is provided a method of
 3 manufacturing a compound as described above, said
 4 method comprising providing an intermediate compound
 5 A and an intermediate compound B,
 6 wherein intermediate compound A has the structure
 7 $R_A M$ wherein M is a metal or metalloid group (such as
 8 $ZnCl$, $SnBu_3$ or $MgBr$) where the metal is directly
 9 attached to R_A , and R_A is a C_5 to C_{30} saturated or
 10 unsaturated alkyl chain which may optionally be
 11 substituted with small alkyl groups such as CH_3 and
 12 C_2H_5 and $R_A M$ is capable of participating in
 13 transition metal catalysed cross-coupling reactions;
 14
 15 and intermediate compound B has the following
 16 structure:
 17



18 wherein;
 19 R_{12} represents OH or an O-protecting group
 20 R_3 , R_{10} , R_{11} , R_{13} , and R_{14} each independently represent
 21 H, OH, a C_1 to C_4 aliphatic alkyl group or an O-
 22 protecting group where required, and optionally
 23 there is a double bond between C_2 and C_3 of the C
 24 ring;
 25

1 and X is a halogen, O-trifluoromethane sulphonate or
2 any other group used in cross-coupling reactions;
3
4 and reacting intermediate compound A with
5 intermediate compound B by transition metal
6 catalysed cross-coupling reactions and subsequently
7 deprotecting at least one OH group.
8 Preferably R_M is an organomagnesium, organozinc,
9 organoboron or organotin compound. Alternatively M
10 may be silyl group.

11
12 The transition metal catalyst may be any suitable
13 transition metal catalyst used in cross-coupling
14 reaction and particular mention may be made of
15 palladium, nickel or iron complexes.

16
17 The protecting group may suitably be methoxymethyl,
18 benzyl (with an optionally substituted aromatic
19 ring) or a small alkyl group such as methyl.

20
21 Usually all of the OH groups will be protected but
22 it may be possible that certain groups need not be
23 protected under certain reaction conditions.

24
25 The present invention will now be further described
26 by reference to the non-limiting examples and figure
27 in which:

28
29 Fig 1 shows the decay curve of the galvinoxyl
30 resonance obtained in ESR timesweep mode (static
31 field) during in situ reduction of the radical by

1 quercetin. Inset is the fieldsweep spectrum of
2 galvinoxyl.

3

4 **Example 1**

5

6 Target 1 (a compound according to the present
7 invention) was synthesised using the reaction shown
8 in Fig 3.

9

10 Target 1 retains potent antioxidant activity. This
11 is defined as the reaction stoichiometry
12 between the target and the synthetic free radical,
13 galvinoxyl.

14

15 In terms of the physico-chemical properties, the
16 compound partitions overwhelmingly into the organic
17 layer of an octanol-water bi-phasic system. This
18 system is a commonly used model of membrane
19 affinity. Target 1 has a high octanol solubility
20 and a long alkyl chain that should allow optimal
21 orientation in lipid membranes. A unique high-
22 potency and strongly lipid-soluble antioxidant was
23 produced.

24

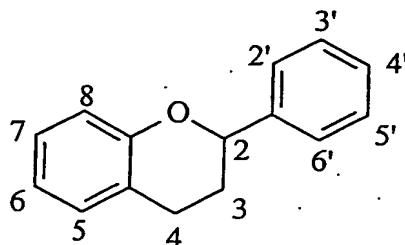
25 The reaction scheme of Example 1 may be repeated
26 using different alkyl chains.

27

28 In all the following examples and discussions, we
29 will use the traditional numbering scheme for
30 flavonoids rather than that defined in Formula 1
31 above. The traditional numbering is as shown below:

32

1



2

3

Example 2.

4

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Background

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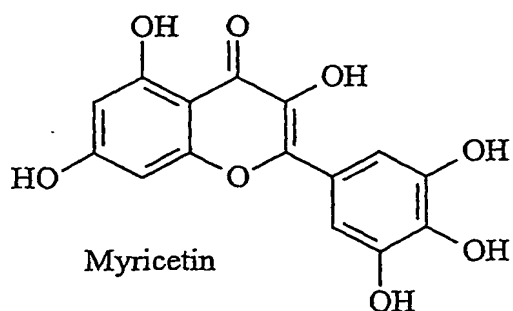
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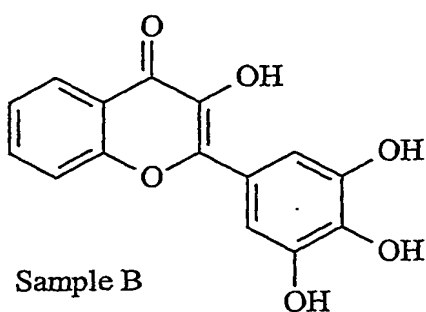
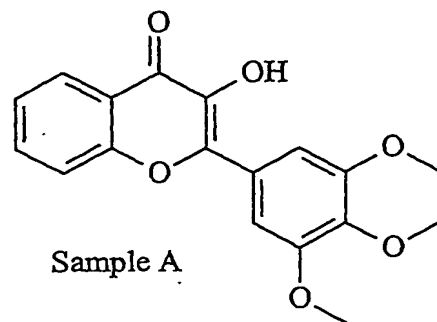
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Inhibition of TBARS production in rat liver microsomes from vitamin E-deficient rats by pre-incubation with target antioxidant and related compounds.

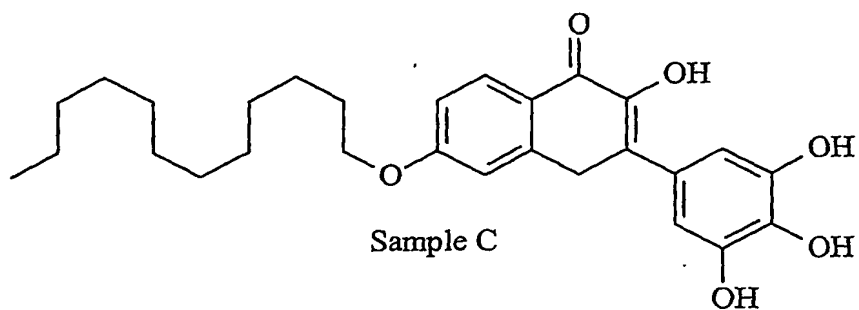
Microsomes are subcellular fractions containing membrane fragments. In vitamin E-deficient rats, microsomes are especially prone to oxidative free radical damage. This can be quantified in terms of the production of thiobarbituric acid reactive substances (TBARS) which result from radical-mediated destruction of the polyunsaturated fatty acid constituents. Consequently, this is a useful biological model to determine the efficacy of phytochemicals as antioxidant membrane protectants. Vitamin E-deficient microsomal suspensions were incubated for 30 minutes with either the target compound (Target 2), myricetin, sample A, sample B, sample C (as shown below) or d alpha-tocopherol.



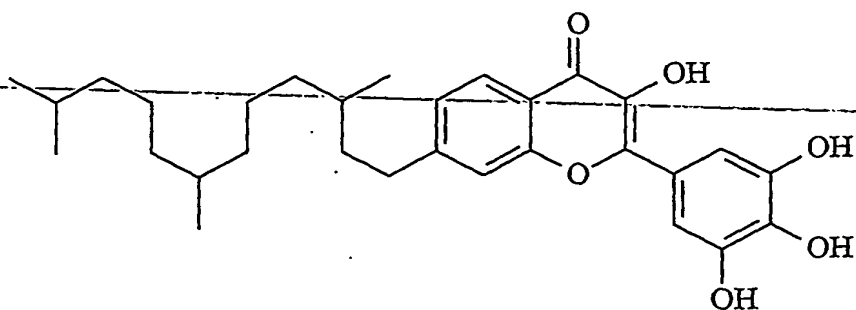
1



2



3



4 The above Target 2 compound was synthesised using
 5 the reaction shown in Figure 4.

6

7 The microsomal suspension was then added to
 8 solutions containing Fe(II)-ADP/ ascorbate to

1 initiate free radical-mediated oxidation and
2 incubated for a further 0, 5, 10, 15 or 20 minutes.
3 TBARS production was then measured by HPLC.

4 5 **Results**

6
7 In the absence of antioxidant protection (-E), TBARS
8 production increases with time. Myricetin (M),
9 although a potent antioxidant in chemical systems
10 affords almost no protection. Compound A, in which
11 the B-ring substituents are methoxylated is non-
12 protective. We have also demonstrated the lack of
13 antioxidant activity in the ESR chemical model
14 system. Sample B, in which the two hydroxyls of
15 myricetin have been removed to increase
16 lipophilicity, is very soluble in octanol, and we
17 have shown by ESR that it retains potent antioxidant
18 activity. However, it does not give rise to
19 significant membrane protective effects. Sample C,
20 which comprises an unbranched alkyl chain linked to
21 the A-ring via oxygen and wherein the alkyl chain
22 length is that of our target molecule, shows
23 efficacy in the initial stages of microsomal
24 oxidation. However, the protection is lost after 20
25 minutes. The target (T) suppresses oxidative damage
26 throughout the 20 minute period and is comparable in
27 effectiveness to α -tocopherol (α). The greater
28 efficacy of Target 2 in comparison to sample B shows
29 that orientation within the membrane is vital to
30 suppressing oxidation, and that lipophilicity alone
31 is not sufficient. The greater efficacy of Target 2
32 in comparison to sample C is probably due to the

1 methylated chain of Target 2 more closely resembling
 2 the side-chain of vitamin E, thus ensuring correct
 3 orientation in the membrane.

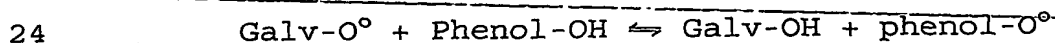
4

5 Example 3

6

7 Within a biological system where a number of
 8 polyphenols may be present at similar
 9 concentrations, antioxidant efficacy may be
 10 predominantly governed by reaction kinetics rather
 11 than stoichiometry. Consequently, the antioxidant
 12 potential of thirteen flavonoids and vitamin E were
 13 assessed and their kinetic and stoichiometric
 14 reduction of a synthetic radical using stopped-flow
 15 electron spin resonance (ESR) spectroscopy has been
 16 compared. The radical used was galvinoxyl (Galv-
 17 O°), (2,6-di-tert-butyl- α -(3,5-di-tert-butyl-4-oxo-
 18 2,5-cyclohexadien-1-ylidene)-p-tolyloxy) which is
 19 resonance-stabilised and sterically-protected, and
 20 so displays little self-reactivity in solution, is
 21 reduced by H-atom transfer reactions in the presence
 22 of phenolic compounds.

23



25

26 The process is governed by the O-H bond dissociation
 27 enthalpy of the donor. Galvinoxyl has a well-
 28 defined ESR spectrum and this property was used to
 29 calculate second order rate constants, as well as
 30 establishing stoichiometry, for the reaction with
 31 phenolic compounds.

32

1 **Materials**

2

3 Tamarixetin and myricetin-3',4',5'-trimethylether
4 were purchased from Indofine Chemical Co.

5 (Somerville, USA). The remaining flavonoids, d- α -
6 tocopherol and galvinoxyl (2,6-di-tert-butyl-a-(3,5-
7 di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-
8 tolyloxy) were purchased from Sigma-Aldrich Chemical
9 Co. (Poole, Dorset, UK) and ethanol (>99.7%) from
10 BDH Laboratory Supplies (Poole, Dorset, UK).

11 Reagents were used without further purification.

12

13 **Methods**

14 *Kinetic Measurements*

15

16 Ethanolic solutions of flavonoid (0.2 mM) and
17 galvinoxyl (0.2 mM) were de-oxygenated under a
18 stream of nitrogen gas. Aliquots (6 ml) were
19 transferred to Hamilton gas-tight syringes (10 ml)
20 coupled to a pneumatic ram and connected to a two-
21 stream ESR quartz flow-cell. *In situ* reaction at
22 20°C \pm 2°C between the flavonoid and galvinoxyl was
23 initiated by rapidly evacuating the syringes.
24 Spectra and decay curves were obtained on a Bruker
25 ECS 106 spectrometer operating at ca. 9.5 GHz (X-
26 band) and equipped with a TM₁₁₀ cavity. Decay curves
27 were obtained by operating in timesweep mode with
28 the static field set at the resonance maximum of the
29 galvinoxyl signal.

30

31

32

Stoichiometric Measurements

Ethanollic solutions of flavonoids (0.1 mM) were prepared. Aliquots (3 ml) of an ethanollic galvinoxyl solution (0.5 mM) were mixed with an equal volume of flavonoid solution then transferred to an ESR quartz cell. The spectra and reaction stoichiometry were evaluated. In brief, the spectra of the unreacted galvinoxyl were obtained 5 minutes from mixing, by which time equilibration was complete. The galvinoxyl concentrations remaining were calculated by double integration of the signal and comparing with the control experiment where ethanol was added to the galvinoxyl solution instead of flavonoid solution.

Results

The ESR spectrum of galvinoxyl in an ethanollic solution consists of a doublet of quintets (Figure 1) which arise from the interaction of the unpaired electron spin with the nuclear spins of the proton on the central carbon and the four equivalent aromatic ring protons. ~~In the presence of a~~ hydrogen donating compound, such as quercetin, the resonances decay as reduction of the radical proceeds. Data from all the decay curves gave a good linear fit to the second-order integrated rate expression, with the average correlation coefficient for each set of replicates being greater than 0.970. However, there were marked differences between the flavonoids in the kinetics of the reduction of the

galvinoxyl free radical (Figure 2). Myricetin and morin were, by far, the fastest to react whereas hesperitin and apigenin showed little reactivity. Ranking of reaction rates as second order rate constants was: myricetin > morin > quercetin > fisetin \approx catechin > kaempferol \approx luteolin > rutin > taxifolin > tamarixetin > myricetin-3',4',5'-trimethylether > datiscetin > galangin > hesperitin \approx apigenin. Reaction rates of eight of the flavonoids were greater than that for vitamin E.

The stoichiometry of the reaction of these compounds with the galvinoxyl free radical was determined by adding the flavonoid, or vitamin E, to an excess of the radical and allowing the reaction to proceed to the endpoint. This resulted in a ranking of antioxidant capacity which differed from the kinetic ranking (Figure 2) i.e. myricetin > fisetin > quercetin \approx luteolin > rutin > catechin > taxifolin > kaempferol \approx morin > datiscetin > tamarixetin > myricetin-3',4',5'-trimethylether \approx galangin > hesperitin > apigenin. In particular, the reaction of morin with galvinoxyl had the second fastest rate of all compounds, but was only ranked eighth equal in terms of the number of radicals reduced. Seven of the flavonoids had a greater reaction stoichiometry than vitamin E. Datiscetin, galangin, hesperitin and apigenin were the four lowest ranked of all the compounds in both the kinetic and stoichiometric measurements of antioxidant potential.

Discussion

A large number of natural phenolic compounds in fruit, vegetables, tea and wines have antioxidant activity due to their hydrogen donor activity and their ability to complex transition metal ions. In addition to the location and total number of hydroxyl groups, the solubility of the phenolics in the test medium may significantly affect their ability to act as antioxidants. For example, antioxidant activity of flavonoids in lard appears to be related to the number of *ortho*-dihydroxy groupings in the A and B-rings whereas a lack of conjugation between the B and C-rings is a major influence in aqueous media. The kinetic measurements in the present Application indicate that reactivity of the flavonoids with galvinoxyl in an organic medium is highly-dependent on the configuration of OH groups on the B and C-ring systems.

Galangin, which has no OH groups on the B-ring reacted only very slowly. However, addition of an OH group to the 4' position (~~position 12 in formula~~ I) (kaempferol) increased the rate by a factor of about 70. The presence of an OH group on the C-ring was also important because the reaction with apigenin, which has the 4'-OH group (position 12 in formula I), but no OH at the 3-position on the C-ring, was slow, whereas the rate of reaction with kaempferol, which has both of these hydroxyl groups, was almost 250-fold greater.

1 The importance of further addition of hydroxyl
2 groups to the B-ring was illustrated when comparing
3 luteolin to apigenin. Luteolin is apigenin with an
4 OH added ortho- to the 4'-OH (position 12 in formula
5 I). The presence of this catechol function imparts
6 significant activity in its own right as luteolin,
7 which lacks the 3-OH, reacted with galvinoxyl at a
8 rate similar to kaempferol. However, the ability of
9 the 3-OH to enhance reactivity was demonstrated by
10 the doubling of the rate constant in quercetin
11 compared with luteolin.

12
13 The difference in rate constant between quercetin
14 and rutin also illustrated the influence that a
15 group at the 3-position has on the kinetics of the
16 reaction of flavonoids with galvinoxyl.

17
18 Substitution of the 3-OH of quercetin by an ether-
19 linked sugar group (rutin) caused an approximate 3-
20 fold decrease in the rate of reaction, although the
21 rate constant was still greater than those for
22 apigenin, hesperitin, galangin, datiscetin,
23 taxifolin and vitamin E. By comparison with
24 luteolin, the increased reaction rate of quercetin
25 may be ascribed to electron donation by the 3-OH
26 through the resonance effect, as the B- and C-rings
27 of the flavonoids are linked by an extended,
28 conjugated, π -electron system. In the case of
29 rutin, despite the electron donating ability of the
30 ether group, the rate is lower than that of
31 luteolin. The importance of conjugation is further
32 highlighted by the 7-fold diminution in rate

1 observed when the C-ring 2,3 bond of quercetin is
2 saturated (taxifolin). More difficult to explain is
3 the activity retained by (+)-catechin which also
4 lacks the 2,3 double bond. Catechin differs from
5 taxifolin by the absence of the C-ring carbonyl
6 group (and use of the single stereoisomer rather
7 than racemic mixture). It may be that the hydrogen
8 of the 3-OH is in close enough proximity to the B-
9 ring to interact and increase the ability of the
10 ring to sustain unpaired electron spin density.
11 Thus a second mechanism to enhance reactivity may
12 operate independent of resonance stabilisation
13 through the 2,3 double bond. With taxifolin, intra-
14 molecular hydrogen bonding of the 3-OH to the
15 carbonyl would inhibit this mechanism and may
16 account for the 5-fold reduction in rate compared
17 with catechin.

18
19 Hydroxylation at the 4' position on the B-ring
20 (position 12 in formula I) was an important feature
21 of reactivity. Comparison of the kaempferol and
22 datiscetin rate constants demonstrated a 56-fold
23 reduction in activity on moving the hydroxyl from
24 ~~the 4' (position 12 in formula I) to the 2' position~~
25 (position 10 in formula I). The presence of a 2'-OH
26 (position 10 in formula I), however, substantially
27 increases the reactivity of a hydroxyl on the 4'
28 position (position 12 in formula I) as evidenced by
29 the 8-fold increase in rate which morin displays
30 relative to kaempferol. Methoxylation of the 4'-
31 position (position 12 in formula I) of quercetin
32 (tamarixetin) resulted in a 15-fold reduction in

1 rate suggesting that the O-H bond dissociation
2 enthalpy at the 4' position (position 12 in formula
3 I) in quercetin is most favourable for H-atom
4 transfer.

5
6 Of the fifteen flavonoids examined, eight had rate
7 constants greater than that of vitamin E.
8 Reaction stoichiometries show that many flavonoids
9 can undergo multiple H-atom, or electron transfer,
10 steps (see Table 1). Most effective in this respect
11 was myricetin, in which each molecule could reduce
12 four molecules of the radical. The non-integer
13 values suggest that inter- or intra-molecular side
14 reactions, involving partially-oxidised flavonoid
15 intermediates, occur. The most important
16 determinant of a high stoichiometric value was the
17 presence of a catechol function on the B-ring. Of
18 the fifteen compounds examined, eight were
19 hydroxylated at the 3' position (position 11 in
20 formula I) and 4' position (position 12 in formula
21 I) and had reaction stoichiometries ranging from 2.8
22 (taxifolin) to 4.1 (myricetin). Without this
23 functional group, the highest activity achieved was
24 1.8 (kaempferol and morin). The enhanced reductive
25 capacity afforded by the catechol moiety is a
26 possible consequence of a two-step oxidation to the
27 ortho quinone. Morin, in which the second B-ring
28 hydroxyl group is placed meta to the 4'-OH (position
29 12 in formula I), and consequently is unable to
30 effect quinone formation, has a stoichiometric value
31 of 1.8 compared with 3.3 for quercetin in which the
32 second hydroxyl is placed ortho to the 4' position

(position 12 in formula I). Activity was not a simple function of the number of hydroxyl groups present on the B- and C- rings. For example, datiscetin is morin with the 4'-OH (position 12 in formula I) removed, yet its reaction stoichiometry is essentially the same as that of morin. Rutin, which is quercetin with the 3-OH replaced by an ether-linked sugar moiety, retains similar activity. A poor correlation ($r = 0.44$) was found between the kinetic and stoichiometric parameters for the reduction of galvinoxyl by flavonoids. In particular, datiscetin, kaempferol and morin had almost identical reaction stoichiometries (ca 1.8), yet the reaction rates were 22, 1243 and 10134 $\text{mol}^{-1} \text{dm}^3 \text{s}^{-1}$, respectively. These results highlight the importance of considering reaction kinetics, as well as stoichiometry, when assessing antioxidant capacity. Where two, or more, potential antioxidants are present, as may occur in complex cellular environments, kinetic factors may greatly over-ride reaction stoichiometry in determining which compound will afford greatest protection. Flavonoids, such as quercetin, may get absorbed from the diet into tissues. Consequently, kinetics and stoichiometry must both be considered in assessing the relevance of plant phenolics as nutritional antioxidants for disease prevention. This ESR method is a useful model to determine these two distinct aspects of antioxidant activity in a non-aqueous environment, as may be encountered in the lipid phase of cells. The galvinoxyl radical is insufficiently oxidising to indiscriminately

1 abstract H-atoms from a wide range of substrates.
2 Therefore, reactions are only likely to be
3 significant with good H-donors, i.e. compounds which
4 may fulfil an antioxidant role within a biological
5 context.

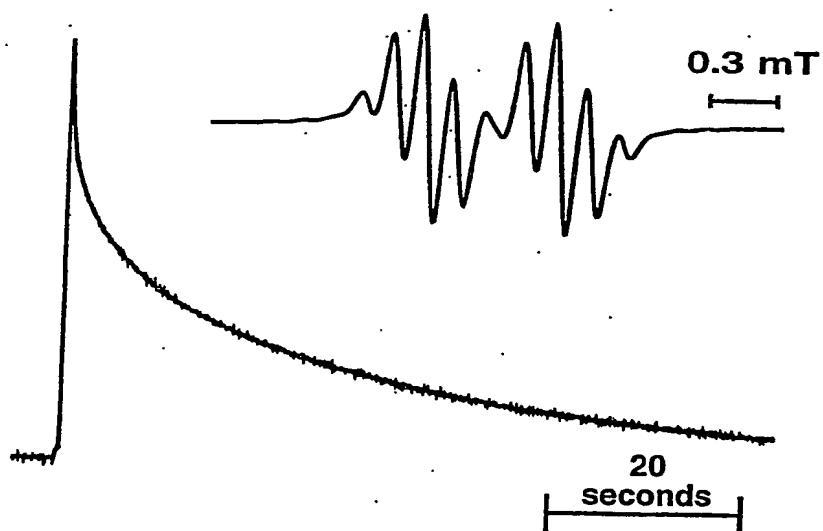
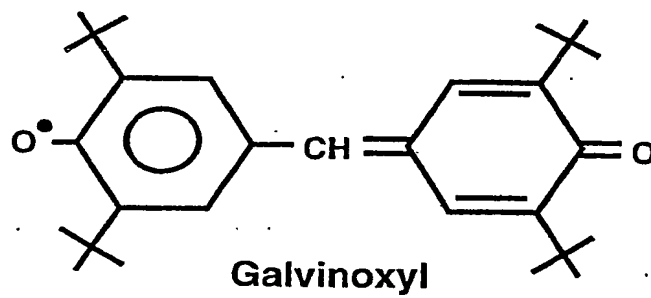
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Substitution Pattern

Compound	k_2	Reaction Stoichiometry	3	4	5	7	2'	3'	4'	5'
Catechin	1574±79	2.96±0.01	-H, OH	-H, H	-OH	-OH		-OH	-OH	
Taxifolin	337±32	2.82±0.05	-H, OH	=O	-OH	-OH		-OH	-OH	
Hesperitin	6±0.5	0.20±0.02	-H, H	=O	-OH	-OH		-OH	-OMe	
Apigenin	5±0.5	0.04±0.02	-H	=O	-OH	-OH		-OH	-OH	
Luteolin	1212±45	3.24±0.01	-H	=O	-OH	-OH		-OH	-OH	
Galangin	18±1	1.01±0.03	-OH	=O	-OH	-OH		-OH	-OH	
Fisetin	1623±199	3.68±0.03	-OH	=O	-OH	-OH		-OH	-OH	
Kaempferol	1243±99	1.84±0.01	-OH	=O	-OH	-OH		-OH	-OH	
Quercetin	2383±258	3.27±0.04	-OH	=O	-OH	-OH		-OH	-OMe	
Tamarixetin	165±20	1.14±0.03	-OH	=O	-OH	-OH		-OH	-OH	-OH
Rutin	670±41	3.18±0.01	-ORut*	=O	-OH	-OH		-OH	-OH	-OH
Myricetin	14463±1767	4.08±0.01	-OH	=O	-OH	-OH		-OH	-OH	-OH
Tri-Ome-Myricetin	74±14	1.06±0.02	-OH	=O	-OH	-OH		-OMe	-OMe	-OMe
Datisetin	22±2	1.74±0.02	-OH	=O						
Morin	10134±459	1.83±0.01	-OH	=O	-OH	-OH	-OH	-OH	-OH	
Vitamin E	524±48	2.14±0.12			-OH	-OH	-OH	-OH		

Second order rate constants (k_2) and reaction stoichiometries for the reduction of galvinoxyl radical by flavonoids and vitamin E. *Rutin is quercetin-3-rutinoside. The compounds above the dotted line are based on the 2-H flavan system, while those below are Δ -2-flavan-4-ones.

Figure 1Figure 2

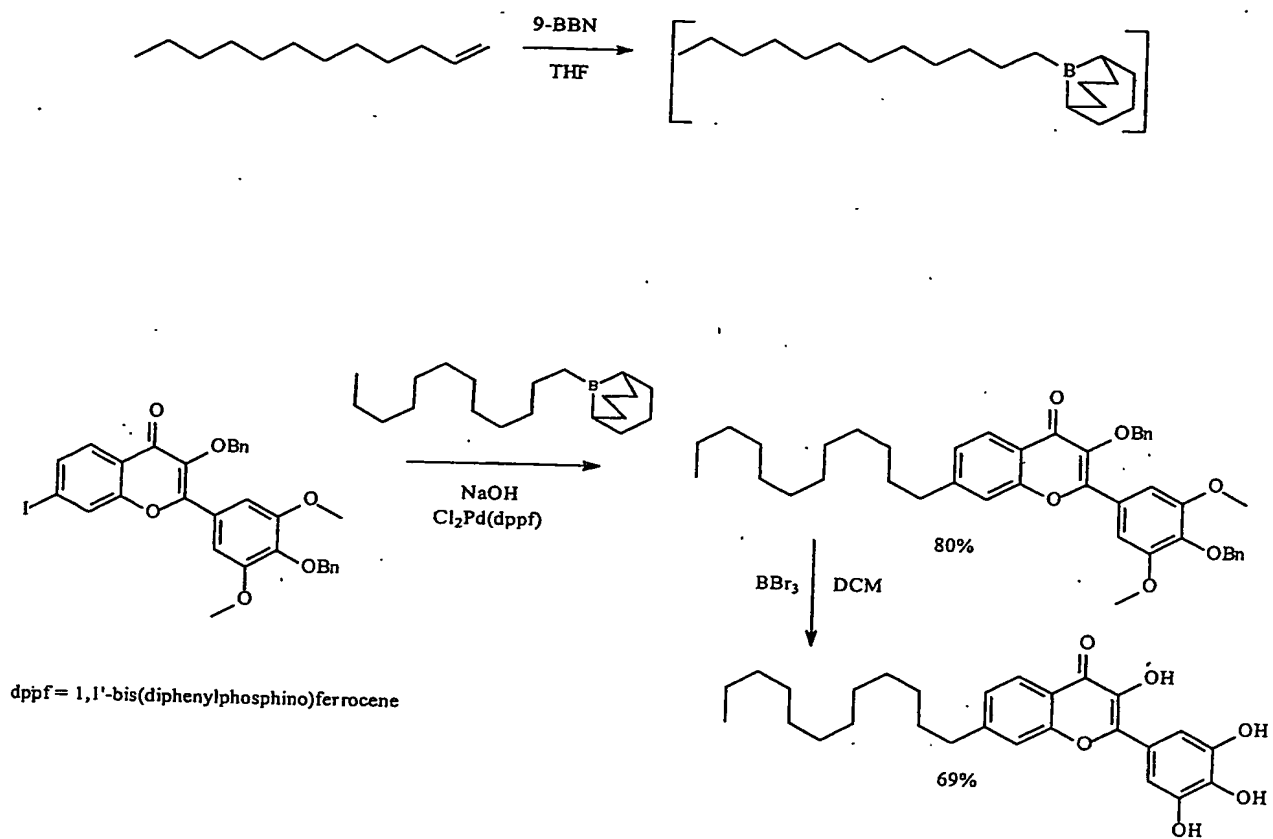


Fig 3

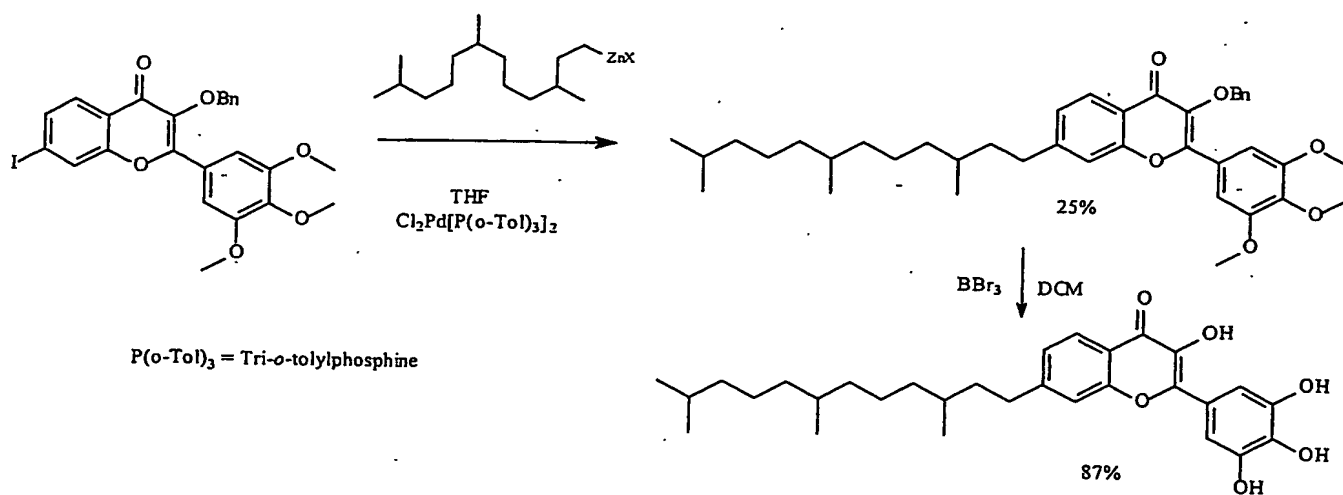
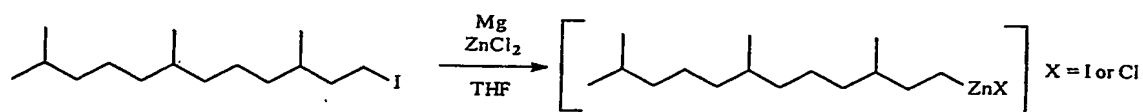
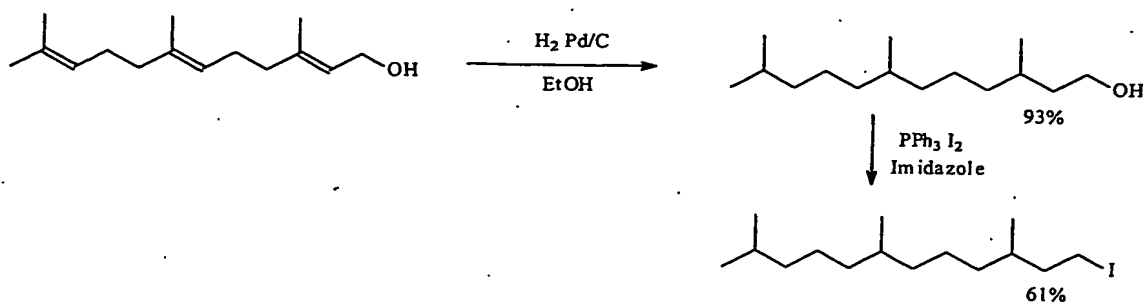


Fig 4

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